Modulating effect of retinoic acid on epithelial differentiation and mucin expression in cultured human corneal limbal epithelial cells

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Directed by Professor Eung Kweon Kim

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<ABSTRACT>

Modulating effect of retinoic acid on epithelial differentiation and mucin expression in cultured human corneal limbal epithelial cells

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Damage to the ocular surface and corneal epithelial stem cells at the limbus from severe cicatricial ocular surface disorders such as chemical and thermal burns or Stevens-Johnson Syndrome has long posed a major challenge to ophthalmologists. The development of new surgical method using ex vivo cultivated epithelial sheets has brought in new era in ocular surface reconstruction. Maintenance of the healthy wet ocular surface is facilitated by the presence of mucins secreted on its surface as well as by the membrane-associated mucins in the apical cell glycocalyx. Given that the mucin layer contributes to epithelial barrier function, maintenance of the mucous phenotype may be a desirable property of transplantable epithelial cell sheets. However, regarding the various corneal epithelial cell culture methods, only a few studies have investigated mucin expression or other functional properties of epithelial cell sheets themselves.

In this study, we sought to find an optimized serum-free condition for producing a fully differentiated corneal epithelial cell sheet with intact barrier function. Thus, we aimed to make our corneal epithelial cells maintain their mucous phenotypes, because membrane-associated mucins have critical roles in

maintaining the healthy ocular surface by providing a barrier to penetration of pathogens.

Retinoic acid (RA), a biological derivative of vitamin A, has been known to be essential for epithelial differentiation and for maintaining mucous phenotype. Thus, RA has been used as an essential element in serum-free culture media. It showed modulating effects on cellular proliferation and differentiation in various epithelial tissues. Thus, we investigated whether RA has a modulating effect on the differentiation of corneal epithelial cells. Furthermore, we investigated the effect of RA on the expression of membrane-associated mucins in the cultured corneal epithelial cells.

Human corneal limbal epithelial (HCLE) cells were dissociated from donor eyes and grown in growth factor supplemented serum-free media. HCLE cells were cultured in RA-deficient or RA-supplemented media with various concentrations (10^{-9} to 10^{-6} M). Passage-3 cells were differentiated using the air-liquid interface technique after cells reached confluence in Transwell culture insert. As a morphologic characterization, overall thicknesses of cell layers and keratin layers were examined by hematoxylin/eosin staining and apical surface was examined by scanning electron microscopy (SEM). Stratified epithelial cells were then examined by immunohistochemistry using differentiation related markers such as p63, cytokeratin 3 (CK3), and MUC16. To examine the epithelial barrier function, HCLE cells were incubated with rose bengal dye. Furthermore, the effect of RA on the expression of three membrane-associated mucins (MUC1, -4, -16) was analyzed by quantitative real-time polymerase chain reaction and western blot analysis in a concentration and time dependent manner.

Histologic and SEM findings suggested that cultured HCLE cells showed features of multi-layered squamous epithelium with numerous microvilli at the apical surface. RA-deficient culture resulted in overproduction of cornified keratin layer, whereas suitable concentrations of RA (10^{-8} to 10^{-7} M) induced

normal appearing non-keratinized squamous epithelium. Under these conditions, p63, CK3, and MUC16 staining patterns were similar to those of limbal corneal epithelium. Higher concentration (10^{-6} M) of RA, however, resulted in fewer-layered epithelial cells with abnormal differentiation patterns. In barrier function test, rose bengal dye was excluded in the stratified epithelial cells grown in RA-supplemented media, whereas, it was easily penetrated in those grown in RA-deficient media. This result implied that cultured epithelial cells expressed functional MUC16. Furthermore, cultured HCLE cells expressed 3 membrane-associated mucins (MUC1, -4, -16) and their expressions were highly associated with RA concentrations and exposure times.

Taken together, our results suggest that RA concentrations in the range of 10^{-8} M seem to be the most appropriate for obtaining functionally differentiated corneal epithelial cells with retaining similar phenotypes to those of limbal corneal epithelium.

In conclusion, this study reported that human corneal epithelial cell was successfully cultured in serum-free, RA-supplemented media. Cultured HCLE cells retained physiologic features of fully differentiated corneal epithelium with barrier function. Our data suggested that such epithelial cells could be an ideal in vitro model for epithelial functional study. Furthermore, they may be suitable for epithelial cell sheet transplantation in ocular surface reconstruction, since they showed similar phenotypes to limbal epithelium. This study also demonstrated that RA was a key modulator in corneal epithelial differentiation and regulates mucin expression in a dose-dependent manner in cultured corneal epithelial cells.

Key words: corneal epithelial cell, membrane-associated mucin, retinoic acid

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I. INTRODUCTION

Severe damage to corneal epithelial stem cells at the limbus has been one of the most challenging conditions to ophthalmologists. In many cases, corneas with limbal deficiency demonstrated conjunctivalization, neovascularization, chronic inflammation, and persistent epithelial defect with associated profound visual loss.[1-3] The development of new surgical method using ex vivo cultivated epithelial cell sheets has brought in a new era in ocular surface reconstruction. This method overcomes the limitations related to conventional limbal transplantation, and the living donor eye is less likely to develop iatrogenic limbal stem cell deficiency. A number of reports have shown encouraging clinical results, although long-term efficacy is yet to be confirmed.[4-9] As an alternative to limited limbal tissues, cultured oral mucosal epithelial cell sheet transplantation has recently been suggested as a promising modality in ocular surface reconstruction.[9-12] Hori and colleagues reported that cultivated oral mucosal epithelial cell sheets express three membrane-associated mucins similar to those in corneal epithelium, and suggested that mucin expression may contribute to the clinical success of cultivated oral mucosal epithelial cell

transplantation for ocular surface reconstruction.[13,14] Given that the mucin layer contributes to epithelial barrier function, maintenance of the mucin production may be a desirable property of transplantable epithelial cell sheets. However, regarding the various corneal epithelial cell culture methods, only a few studies have investigated mucin expression[15-17] or other functional properties of epithelial cell sheets themselves.[18,19]

The most common method for in vitro expansion of limbal epithelial cell involves the use of growth-arrested murine fibroblast feeder layer and fetal bovine or calf serum supplemented media.[9] With growing concern regarding the risk of transmission of zoonotic infection, a demand for culture methods avoiding the use of animal cells or animal serum is gaining interest in regenerative medicine.[20,21] Furthermore, the serum-free culture method provides more suitable in vitro model for studying on cellular differentiation, regulation, or epithelial functions. Therefore, we aim to develop a new culture model, which uses neither a serum nor a murine fibroblast feeder layer. Recently, our group developed a serum-free culture method for human conjunctival epithelial cells using growth factor supplemented culture media and the air liquid interface (ALI) culture technique. With this method, a cultured conjunctival epithelial cells had shown similar differentiation and functional characteristics to those seen in vivo.[22] We, therefore, modified this culture method to make sufficiently stratified and well differentiated corneal epithelial cell sheets with similar phenotypes to those seen in vivo corneal epithelium.

To do this, we hypothesized that retinoic acid (RA), a biologically active derivative of vitamin A, modulates corneal epithelial cellular differentiation. Vitamin A and its derivatives (retinoids) are known to regulate cell proliferation, differentiation, and morphogenesis. The hypothesis that retinoids control epithelial differentiation has its roots in studies on the rat eye showing that its deficiency leads to an increased keratinization of the corneal epithelium.[23,24]

Under vitamin A deficient conditions, columnar respiratory tract epithelium with mucous phenotypes are replaced by a keratinized stratified epithelium similar to epidermis.[25,26] These reports were followed by the important observation that not only vitamin A deficiency, but also vitamin A excess result in epithelial alteration.[27,28] These findings suggest that the effects of vitamin A on modulating epithelial proliferation and differentiation are largely concentration-dependent.

In this study, we sought to find an optimized serum-free condition for producing fully differentiated corneal epithelial cells with intact barrier function. Thus, we intended to make our corneal epithelial cells maintain their mucous phenotypes similar to those seen in native corneal epithelium, because membrane-associated mucins have critical roles in maintaining the healthy wet ocular surface by providing a barrier to penetration of pathogens.[15-17,29,30] To do this, we investigated the modulating effect of RA on the epithelial differentiation and the expression of membrane-associated mucins in cultured human corneal limbal epithelial cells.

II. MATERIALS AND METHODS

1. Human corneal limbal epithelial (HCLE) cell culture model

Fresh human corneoscleral tissues (< 10 days after death) were obtained from Yonsei university Eye bank (Seoul, Korea) after the central corneal buttons were used for corneal transplantation. After removal of excessive tissue, the limbus was immersed in 1.2 U dispase II (Sigma-Aldrich, St Louis, MO, USA) in DMEM/F12 (Gibco, NY, USA) at 4℃ for 12 hours. The corneal epithelial cell sheets were separated from the stroma and isolated epithelial cells were washed three times and suspended in DMEM/F-12 supplemented with 1% penicillin-streptomycin (Gibco) and 10% fetal bovine serum (FBS; Gibco). Cells were preplated on 60 mm culture dishes for 1 hour at 37℃ in a humidified 5% CO_2 atmosphere in order to eliminate fibroblasts by differential attachment. Suspended epithelial cells were seeded at a density of $1x10^3$ cells/cm^2 on 60 mm plastic culture dishes in bronchial epithelial growth medium (Lonza, Walkersville, MD, USA) with growth supplements of defined composition. The growth supplements consisted of insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), epinephrine (0.5 μg/ml), retinoic acid (10 ng/ml), transferrin (10 ng/ml), triiodothyronine (6.5 ng/ml), bovine pituitary extract (0.13 mg/ml), gentamicin:amphotericin (50 μg/ml : 50 ng/ml), all supplied by Lonza and further supplemented with human epidermal growth factor (EGF; Simga-Aldrich, 10 ng/ml), and bovine serum albumin (BSA; Simga-Aldrich, 0.15 mg/ml). The culture medium was changed 1 day after seeding and every other day thereafter until cultures reached 70% confluency. Subcultures were seeded on 100 mm culture dishes at 2 x 10^3 cells/cm^2. Cells not used for

reestablishing cultures were suspended at 1 – 2 x 10^6 cells/ml in culture medium containing 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored frozen in liquid nitrogen for future use. Passage 2 cultured epithelial cells (1 x 10^5 cells in 0.5 ml, 2 x 10^4 cells/cm^2) were seeded onto 24 mm polyestermembranes, 0.4 μm pore size, with Costar Transwell-clear 3450 culture inserts (Corning, NY, USA) in a 1:1 mixture of BEGM:DMEM containing the same supplements as above, except that retinoic acid was added at the desired concentrations (0, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M), and epidermal growth factor was used at 0.5 ng/ml. The cultures were grown submerged for the first 7 days, during which time the culture medium was changed on the first day and every other day thereafter. On day 8, when the cells reached confluence, air liquid interface (ALI) cultures were created by removing the apical medium and feeding the cultures daily only from the basal compartment. All cultures were maintained at 37℃ in a humidified 5% CO_2 atmosphere, and further experiments were conducted on day 14.

2. Retinoic acid preparation

All trans-retinoic acid (RA; Sigma-Aldrich) was dissolved in DMSO and stored in light protective vials at -20℃ until needed. All manipulations concerning the preparation, storage and addition of RA were performed in a dark room. The final DMSO concentration in the medium was never greater than 0.01%, and DMSO alone was also diluted 1:10,000 in RA-deficient culture media as a control. Various dilutions of the RA stock solution (10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M) were freshly prepared in the culture medium after cell seeding and each time the media were changed. To investigate the role of RA on corneal epithelial differentiation and on mucin expression, RA was added at the given concentration to the culture medium when the ALI culture was created, and

differentiated for 7 days. To examine changes in expression of mucin genes following RA treatment on RA-deficient cultures, passage 3 cells were grown for 14, 11, 7, and 0 days in RA-free medium, and then treated with 10^{-8} M of RA for 0, 3, 7, and 14 days, respectively.

3. Morphology evaluation

Live proliferating cells were examined daily by phase contrast light microscopy. After 7 days in stratification, cultured epithelial cells were fixed with 4% paraformaldehyde, embedded in paraffin, cut into 4 μm sections, and stained with hematoxylin/eosin (H/E). The thickness of cornified keratin layer was measured using an imaging analysis program (Image-Pro® Plus version 4.1 for Windows, Silver Spring, MD, USA). For scanning electron microscopy (SEM) comparison, RA-deficient and RA-supplemented cultures were fixed with chilled 2.5% glutaraldehyde for 24 hours, washed with 0.1 M phosphate buffered saline (PBS, Gibco), and postfixed with 1% osmium tetroxide for 2 hours. After critical point drying and gold coating (300 μm thickness), the samples were examined by SEM (H-800, Hitachi, Japan).

4. Immunohistochemistry

For immunohistochemistry, deparaffinized sections were reacted with anti-p63 (4A4, Dako, Carpinteria, CA, USA), cytokeratin 3 (AE5, Santacruz, CA, USA), MUC1 (anti-Mucin 1, AbFRONTIER, Korea), MUC4 (1G8, Zymed, CA, USA) and MUC16 (OC125, DAKO). After washing with TBS, sections were incubated with biotinylated anti mouse IgG and avidin biotinylated peroxidase complex according to the manufacturer's protocol (ABC kit, Vector Laboratory, Burlingame, CA, USA). The samples were then incubated with

3,3'-diaminobenzidine (DAB) substrate to give a brown stain and counterstained with hematoxylin. All experiments were performed three times in duplicate.

5. Rose bengal dye penetrance assay

To examine the epithelial barrier function, HCLE cells grown with different concentrations of RA in culture media were tested for rose bengal dye penetrance. The stratified epithelial cultures were washed three times with PBS, followed by a 5-minute incubation of 0.1% rose bengal dye in calcium and magnesium free PBS. The rose bengal dye was then aspirated and the cell layer photographed using an inverted microscope (Eclipse TS 100; Nikon, Tokyo, Japan). The area of islands of stratified cells that excluded rose bengal was quantified in culture images using an imaging analysis program (Image-Pro® Plus version 4.1 for Windows).

6. Total RNA extraction, Reverse transcription, and Quantitative real-time Polymerase chain reaction

Total RNA from cells was isolated using Trizol reagent (Invitrogen, Rockville, MD, USA), according to the manufacturer's protocols. RNA was digested with DNase I (Invitrogen) before reverse transcription (RT) to remove any residual genomic contamination. Total RNA (3 μg) was reverse transcribed using the first-strand synthesis system for RT (SuperScript II Reverse Transcriptase, Invitrogen) and random hexamer primers according to the manufacturer's instructions. The relative amounts of three mucin mRNAs were analyzed by real-time polymerase chain reaction. Primers used in this study had been previously published (MUC1, MUC16),[17,31] were designed with assistance of computer software (MUC4, Takara, Shiga, Japan), or were purchased from

Applied Biosystems (glyceraldehydes 3-phosphate dehydrogenase, GAPDH). All primers were selected from areas flanking the tandem repeat domains. BLASTN searches against nucleotide databases were performed to confirm the sequence specificity of the chosen nucleotide sequences. Conventional RT-PCR experiments were performed to confirm that a single band was obtained. The real-time PCR was performed three times in duplicate using the StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The reaction condition consisted of a 20 μl reaction volume containing 200 ng of cDNA, 1 μl of primers, and 10 μl SYBR-Premix Ex Taq™ (Takara). The thermocycler parameters were 95℃ for 10 seconds, followed by 40 cycles of 95℃ for 5 seconds and 60℃ for 30 seconds. Non-template controls were included in each assay to confirm lack of DNA contamination. The results were analyzed by the comparative threshold cycle method and normalized by GAPDH. For relative quantification, the amount of mRNA for each target gene was expressed relative to the amount present in the calibrator sample (RA-deficient culture). Results were represented as the mean ± SD of three duplicated independent experiments. Statistical analysis of the differences of expression between RA concentrations was performed with Kruskal-Wallis test and Mann-Whitney U test using SPSS window version 15.0 (SPSS, Chicago, IL, USA). A p-value less than 0.05 was considered to be significant.

7. Western blot analysis

Protein was extracted from cultured corneal epithelial cells with RIPA buffer plus protease inhibitor cocktail. Protein concentration was determined with BCA Protein Assay Reagent kit (Pierce, Rockford, IL, USA). Twenty micrograms of total protein were separated under reducing conditions on 4% stacking and 10% separating sodium dodecyl sulfate (SDS)-polyacrylamide gels, and transferred to PDVF membranes (Millipore Corp., Bedford, MA, USA).

The membranes were blocked with 5% (w/v) nonfat-milk in TTBS (Tris-buffered saline-0.1%Tween20) for 1 hour and incubated first with primary antibodies against MUC1 (anti-Mucin 1, AbFRONTIER), MUC4 (1G8, Zymed), and MUC16 (OC125, Dako), respectively, overnight at 4℃. After washing, membranes were treated with horse radish peroxidase-conjugated secondary antibodies. Protein bands were detected with enhanced chemiluminescence (ECL, GE Healthcare) detection reagents, and then exposed on film. ß-actin was used as an endogenous reference for quantification. Band intensities were quantified with an Image J for Windows (NIH, Bethesda, MD, USA).

III. RESULTS

1. RA improved morphology of cultured corneal epithelial cells

Confluent corneal epithelial cells were small, polygonal, and cobble stone in appearance. After air-lifting, these cells became stratified. The histological examinations showed that cultures produced 2-3 layer thickness epithelial cells after 3 days of air-lifting, and 5-6 layers after 1 week of air-lifting (14 days after plating). Stratification increased until 3 weeks after plating, but severe desquamation was observed in histologic examinations, and cells began to slough off the membranes in large numbers. Based on these observations, 5-6 layered epithelial cells grown for 14 days were selected for use in subsequent experiments.

SEM imaging of the cells grown in RA-supplemented media (10^{-8} M) showed a continuous layer of flat polygonal cells tightly attached to each other. The cell junctions were well developed and the apical surface covered with numerous microvilli. The RA-deficient culture, however, did not have a well-formed cell layer of healthy appearance. It seemed as if the apical cellular structures, if they had ever been present, had been removed, with only the basal portion remaining (Fig. 1).

In order to better understand the respective roles of RA in epithelial cell morphogenesis, the overall architecture of epithelial cultures were compared at different RA concentrations. The epithelial architecture, as judged by the overall thicknesses of the cellular layers and cornified keratin layers, improved as a function of RA. RA-deficient cultures showed hyperkeratotic appearance, as shown by the excessive thickness of the cornified keratin layers (mean $\pm$ SD, 33 $\pm$ 9.5 μm). However, the thickness of the cornified layers significantly decreased after addition of RA, and was hardly detectable in cultures grown at 10^{-8} M or higher concentrations of RA. RA also improves epithelial

morphogenesis. As compared to the RA-deficient cultures, cells grown under suitable concentrations of RA (10^{-8} to 10^{-7} M) produced overall 50 μm thick and well differentiated epithelial cell sheets, indicated by uniform stratified layers with morphologic polarity from basal to superficial layer: cuboidal basal cells, suprabasal wing cells, and flat squamous superficial cells. Furthermore, an excess of RA (10^{-6} M) induced abnormal differentiation, evidenced by the appearance of flat apical cells at suprabasal layers, and reduced overall thickness (Fig. 2).

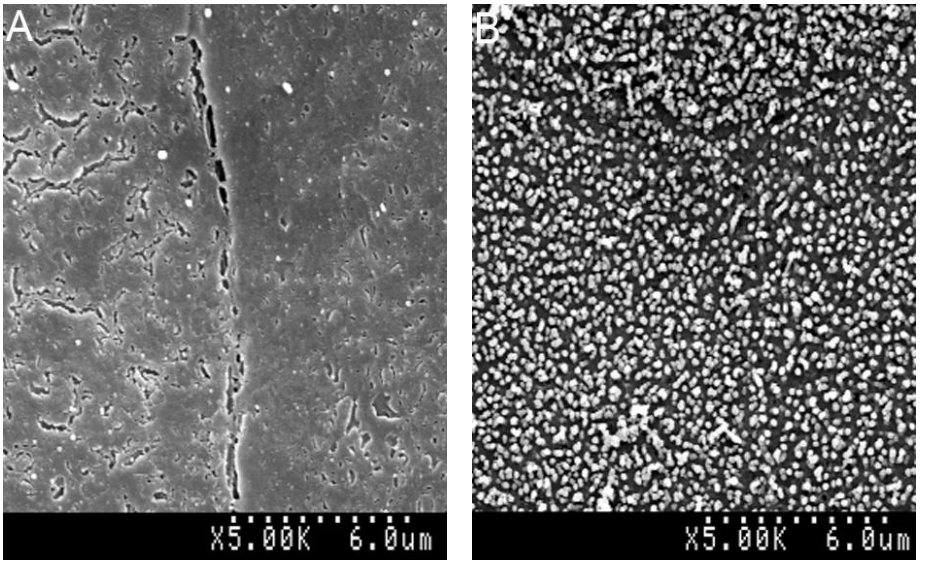

Figure 1. Scanning electron microscopic images of corneal epithelial cells cultured in retinoic acid (RA)-deficient and RA-supplemented media. (A) Cells grown in RA-deficient media did not show well-formed apical structures, whereas (B) those grown in RA-supplemented media (10^{-8} M) were covered with numerous microvilli.

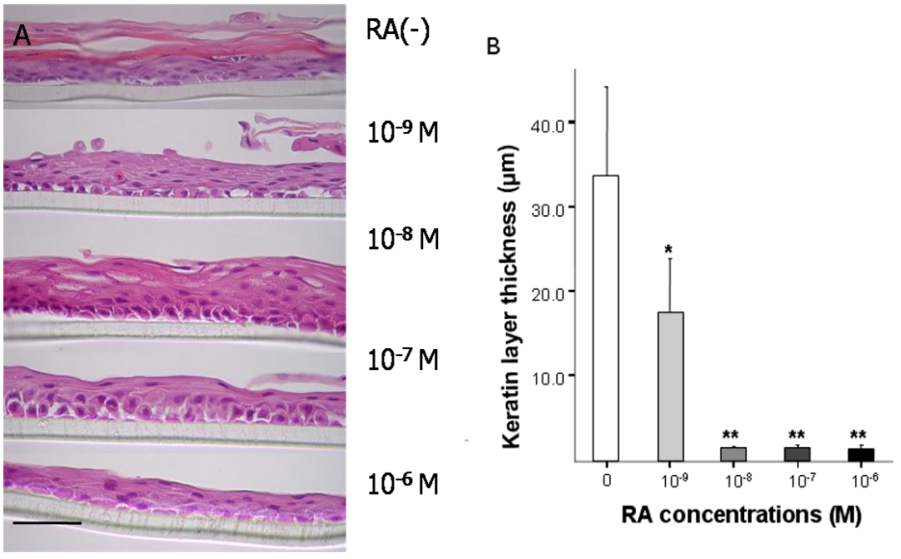

Figure 2. Hematoxylin and eosin staining of cultured corneal epithelial cells and comparison of keratin layer thickness. (A) Stratified corneal epithelial cells grown in RA-deficient media showed hyperkeratotic appearance, whereas the cornified layer significantly disappeared after addition of 10^{-8} M or higher concentration of RA. RA also improved epithelial morphogenesis as indicated by well differentiated appearance in cells grown at 10^{-8} M. Scale bar, 50 µm. (B) RA-deficient cultures showed hyperkeratotic appearance with an average of 33 µm thick keratin layer. The thickness of keratin layers significantly decreased after addition of RA. (n = 5, *: $p < 0.05$ and **: $p < 0.001$ as compared to RA-deficient culture).

2. RA modulated corneal epithelial differentiation

In order to investigate the roles of RA in epithelial cell differentiation, the presence and distribution of stage specific markers were compared at different RA concentrations. A proposed limbal basal cell marker (p63), a cornea-specific differentiation marker (CK3), and a mucinous differentiation marker (MUC16) were chosen. Figure 3 shows representative staining patterns

of p63 and CK3 observed in each RA concentration group. A limbal basal cell marker (p63) was positive in the nucleus of most basal cells and some suprabasal cells in all cultured conditions. CK3 expression was most prominent in cells grown at 10^{-8} M RA, with staining in the suprabasal and superficial layers in most cells. This cornea-specific epithelium marker was also expressed in fair amounts in cells grown at 10^{-7} M RA, but expressed only at superficial layers in lack (10^{-9} M) or excess of RA (10^{-6} M) conditions (Fig. 3).

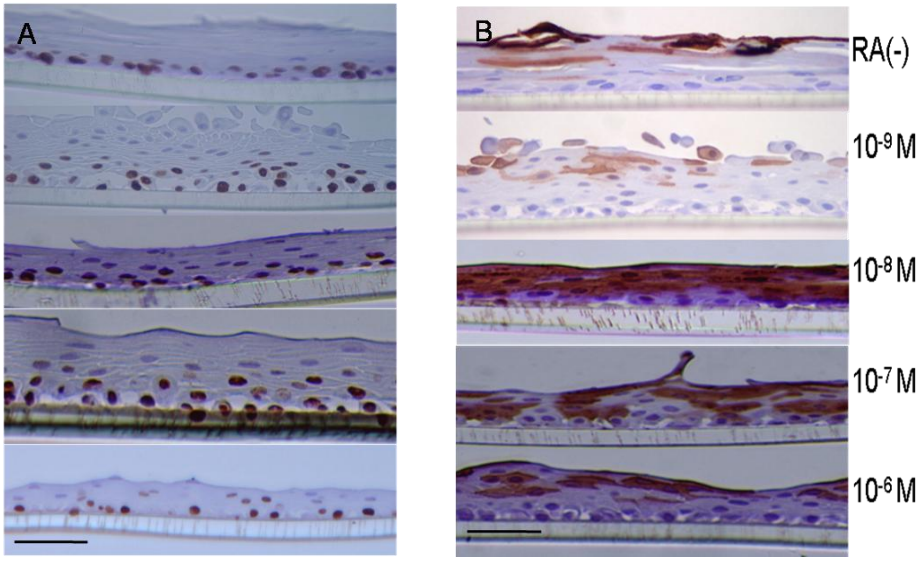

Figure 3. Expression of p63 and CK3 by stratified corneal epithelial cells cultured in different concentrations of retinoic acid. (A) All cultured corneal epithelial cells expressed p63 at basal and suparal basal layers, and no significant differences in staining pattern were noted. (B) Corneal epithelial cells grown at 10^{-8} M RA constantly expressed CK3 in suparabasal cell layer. This cornea epithelium specific marker was expressed also in fair amounts in cells grown at 10^{-7} M RA, but not readily expressed in cells grown in lack or excess of RA. Scale bars, 50 μm.

A mucous marker, MUC16 expression increased with increasing RA concentrations as shown in Figure 4. The stratified epithelial cells cultured in 10^{-6} M RA expressed MUC16 in the whole layer except basal cells, whereas those cultured in 10^{-8} M RA expressed it in apical and subapical layers in a similar pattern to that of normal corneal epithelial cells. Comparison of the immunostaining results of epithelial differentiation related markers and membrane-associated mucins between human corneal limbal epithelium and cultured epithelial cells were summarized in table 1 and 2, respectively.

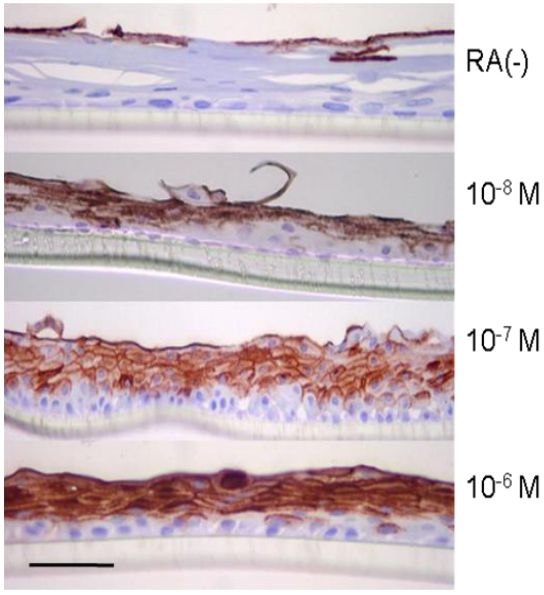

Figure 4. Expression of MUC16 by cultured corneal epithelial cells grown in different concentrations of retinoic acid. Cultured corneal epithelial cells grown at 10^{-8} M RA expressed MUC16 at apical layer, whereas those grown in higher concentrations of RA expressed it also at suprabasal layer. Scale bar, 50 μm.

Table 1. Immunohistochemical results of differentiation related markers in human corneal limbal epithelium and cultured corneal epithelial cells grown in different concentrations of retinoic acid

	Limbal epithelium		Cultured epithelial cells grown with RA (M)							
			0		10^{-8}		10^{-7}		10^{-6}	
Location	p63	CK3	p63	CK3	p63	CK3	p63	CK3	p63	CK3
Apical	-	++	-	+	-	+++	-	+++	-	++
Suprabasal	+	+++	+	-	+	+++	+	++	+	-
Basal	+++	-	+++	-	+++	-	+++	-	+++	-

RA: retinoic acid

+++: strong positive, ++: moderate positive, +: weak positive,

±: ambiguous, -: negative

Table 2. Comparison of immunostaining results of three membrane-associated mucins in human corneal limbal epithelium and cultured corneal epithelial cells grown with 10^{-8} M of retinoic acid

	Limbal epithelium			Cultured epithelial cells[1]		
Location	MUC1	MUC4	MUC16	MUC1	MUC4	MUC16
Apical	+++	++	+++	+++	+	+++
Suprabasal	±	+	±	±	+	+
Basal	-	++	-	-	+	-

[1]: Corneal epithelial cells were cultured in RA-supplemented media (10^{-8} M)
RA: retinoic acid
+++: strong positive, ++: moderate positive, +: weak positive,
±: ambiguous, -: negative

3. HCLE cells cultured in RA-supplemented media excluded rose bengal dye

Rose bengal dye was previously shown to be excluded by cultivated human corneal epithelial cell lines that express MUC16.[29,30] Rose bengal dye penetrance was thus tested in order to examine whether cultivated epithelial sheets express MUC16 on the microplicae structure at the apical surface, and if the barrier function was maintained in serum-free cultivated epithelial cells. In HCLE grown in RA-deficient media, addition of rose bengal resulted in rapid staining of cytoplasm and nucleus. In stratified epithelial sheets grown in RA-supplemented media, however, rose bengal dye did not penetrate islands of stratified cells. Significantly fewer islands excluding the dye were seen in stratified epithelial cells grown in RA-deficient media as compared to those in RA-supplemented media (Fig. 5).

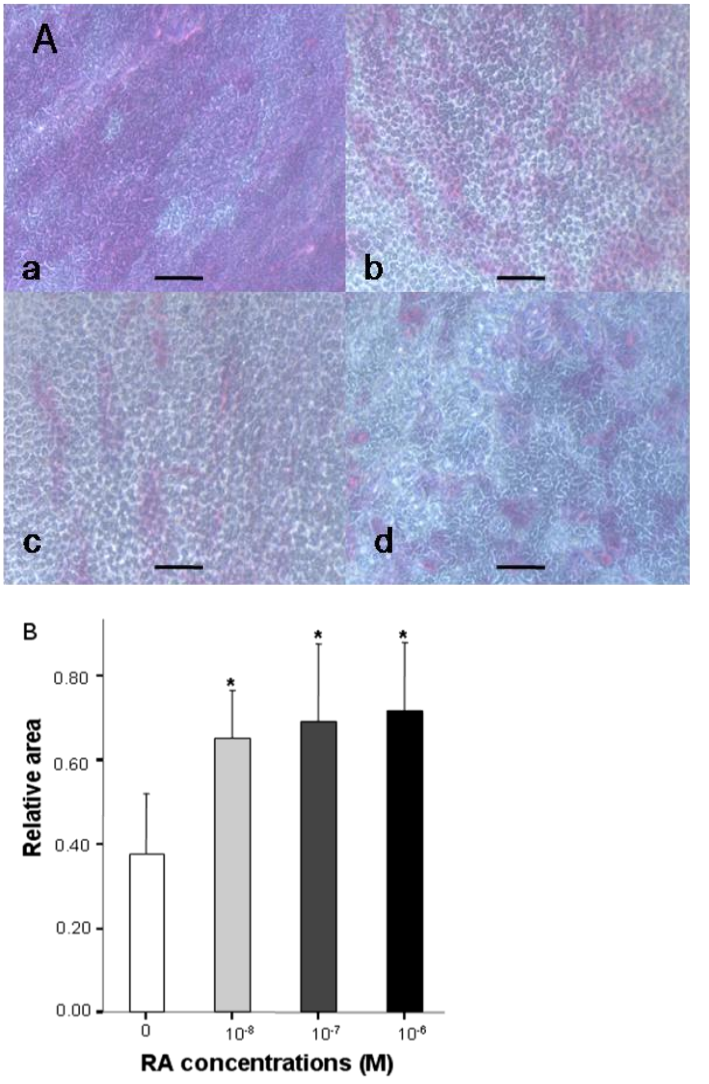

Figure 5. Rose bengal staining in corneal epithelial cells cultured in various concentrations of retinoic acid. (A) Corneal epithelial cells cultured in RA-deficient media (a) were mostly stained with rose bengal dye, whereas stratified cells cultured in RA-supplemented media (10^{-8}, 10^{-7}, and 10^{-6} M; (b), (c), and (d), respectively) were not readily stained with rose bengal dye. Scale bars, 10 μm. (B) Areas of islands excluding rose bengal dye were significantly larger in cells grown in RA-supplemented media as compared to those in RA-deficient media. ($n = 4$, *: $p < 0.05$ as compared to RA-deficient culture).

4. RA increased membrane-associated mucin gene expression

The relative amounts of MUC1, -4, and -16 mRNA were determined by real-time PCR in stratified corneal epithelial cells cultured in medium with various concentrations of RA. Expressing mRNA levels relative to the level of each mucin gene in RA-deficient cells allowed convenient comparisons of the effect of RA concentration on the individual mucin genes. MUC4 mRNA was not readily present in RA-deficient cultures, while MUC1 and -16 mRNA were present in similar amounts. As shown in Figure 6, MUC4 mRNA was significantly induced by RA ($p < 0.05$), and the amount continued to increase in a concentration dependent manner from 10^{-8} to 10^{-6} M. The expression of MUC1 and -16 mRNA in RA-supplemented cultures also increased significantly compared to those of RA-deficient ones ($p < 0.05$). No significant change was detected in MUC1 mRNA levels among different concentrations of RA, whereas the level of MUC16 mRNA was significantly different in 10^{-8} M RA cultures compared to 10^{-7} M and 10^{-6} M RA cultures ($p < 0.05$).

To investigate the effect of RA in a time-dependent manner, cultures were treated with 10^{-8} M RA for different periods. All cultures were grown for 14 days in 24 mm culture insert (7 days under ALI conditions), and RA was added on days 0, 7, and 11. Figure 7 shows the relative expressions of mucin mRNA in cells treated with 10^{-8} M RA for different periods. Mucin mRNA levels were expressed relative to each mucin mRNA level in RA-deficient cells (i.e. cells grown in RA-deficient medium for 14 days). All 3 mucin mRNAs were upregulated after addition of RA to culture medium, and the changes showed time dependency. The amount of changes and the time dependency were different among 3 mucins, with MUC4 mRNA most prominently induced after RA addition, followed by MUC1 and -16. MUC1 mRNA increased as RA treatment times increased, while MUC16 and -4 mRNA expressions were not different between 7 and 14 days of RA treatment, implying that RA was mainly related to differentiation (Fig. 7).

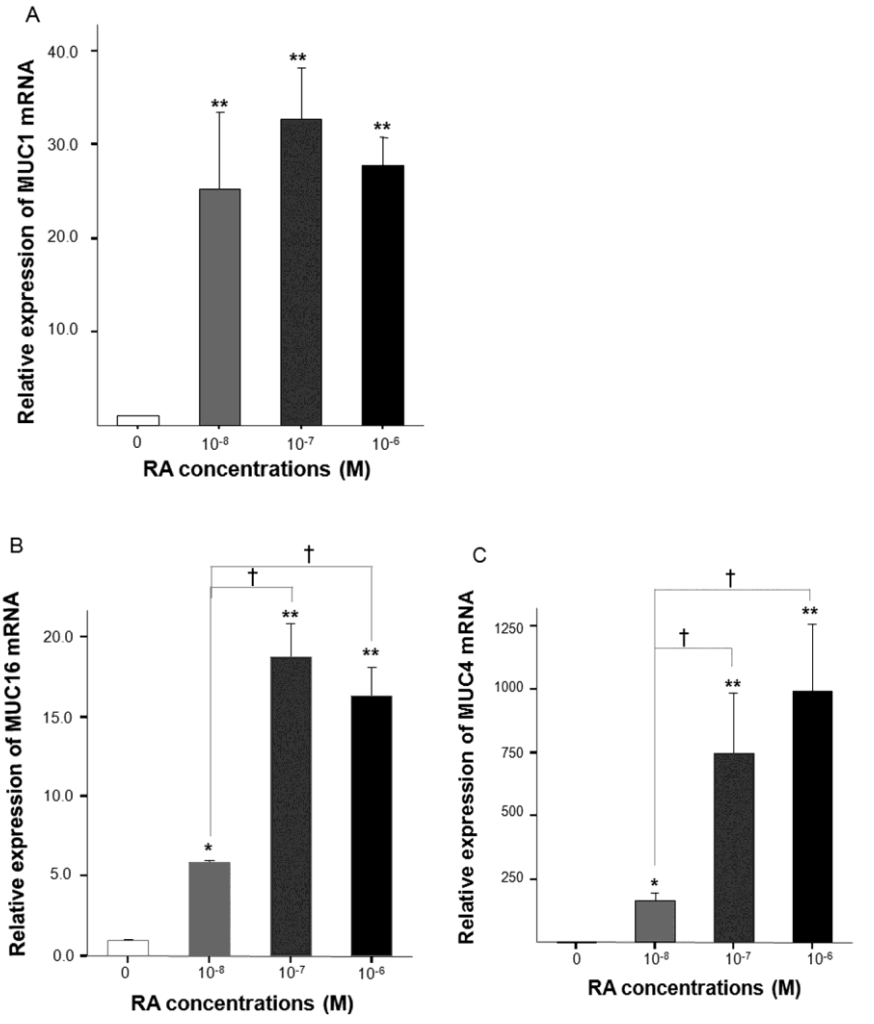

Figure 6. Real-time PCR analysis of MUC1, -16, and -4 expressions in corneal epithelial cells cultured in various concentrations of retinoic acid. (A) MUC1 expression was induced significantly after addition of RA without significant differences among three different RA-supplemented cultures. (B) MUC16 and (C) MUC4 showed similar concentration-dependant induction patterns after addition of RA. (n = 3, *: $p < 0.05$, **: $p < 0.01$ as compared to RA-deficient culture, and †: $p < 0.05$ in comparison between two defined conditions).

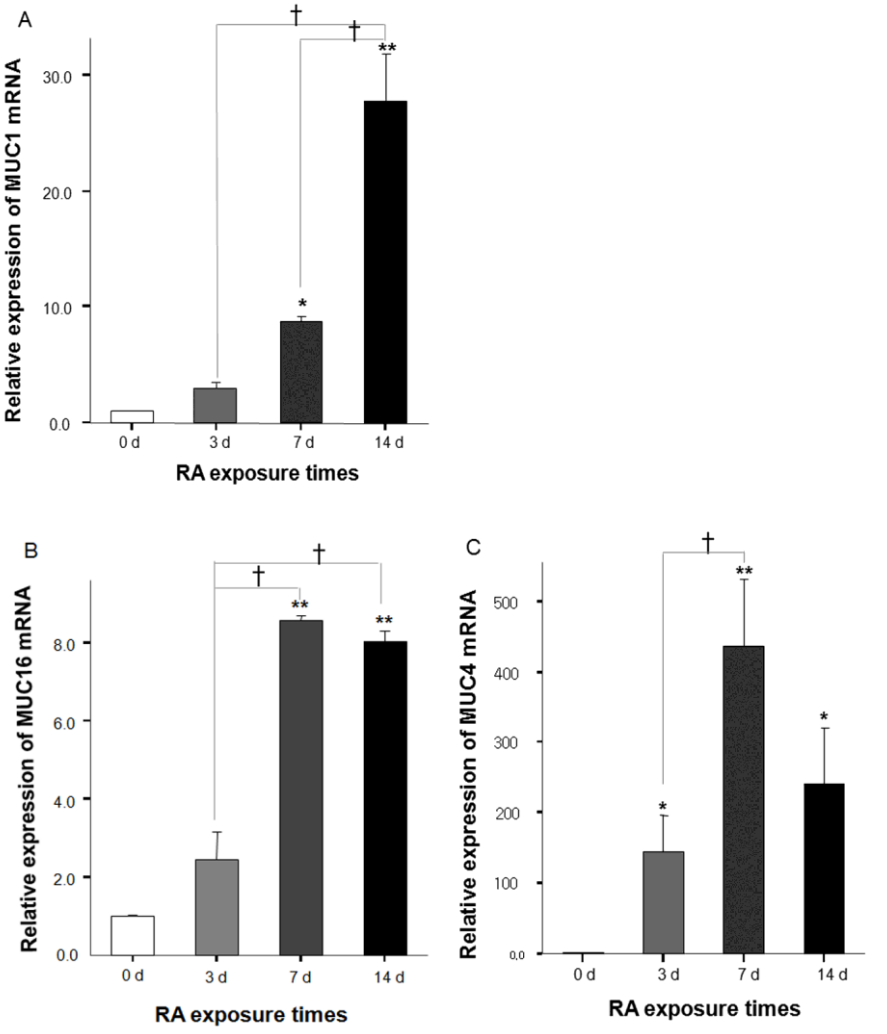

Figure 7. Real-time PCR analysis of MUC1, -16, and -4 expressions in cultured corneal epithelial cells treated with 10^{-8} M of retinoic acid for various times. (A) MUC1 expression increased as RA treatment times increased. (B) MUC16 and (C) MUC4 showed similar induction patterns, in which maximal expressions were reached in 7 days of RA treatment. (n = 3, *: $p < 0.05$, **: $p < 0.005$ as compared to RA-deficient culture, and †: $p < 0.05$ in comparison between two defined conditions).

5. RA increased membrane-associated mucin protein expression

Western blot analyses of MUC 1, -4, and -16 were performed to determine the effect of RA on the protein production of these mucins. Figure 8 shows the immunoblots and densitometric analyses of mucin protein expression in HCLE cells grown at different concentration of RA. Densitmometric comparisons of mucins expression normalized to ß-actin were obtained at different conditions. MUC1 protein levels were increased after addition of RA, however, no significant difference were noted among different concentrations. MUC4 and -16 proteins were not readily observed in RA-deficient cultures, and increased significantly in a concentration-dependent manner in RA-supplemented cultures (Fig.8).

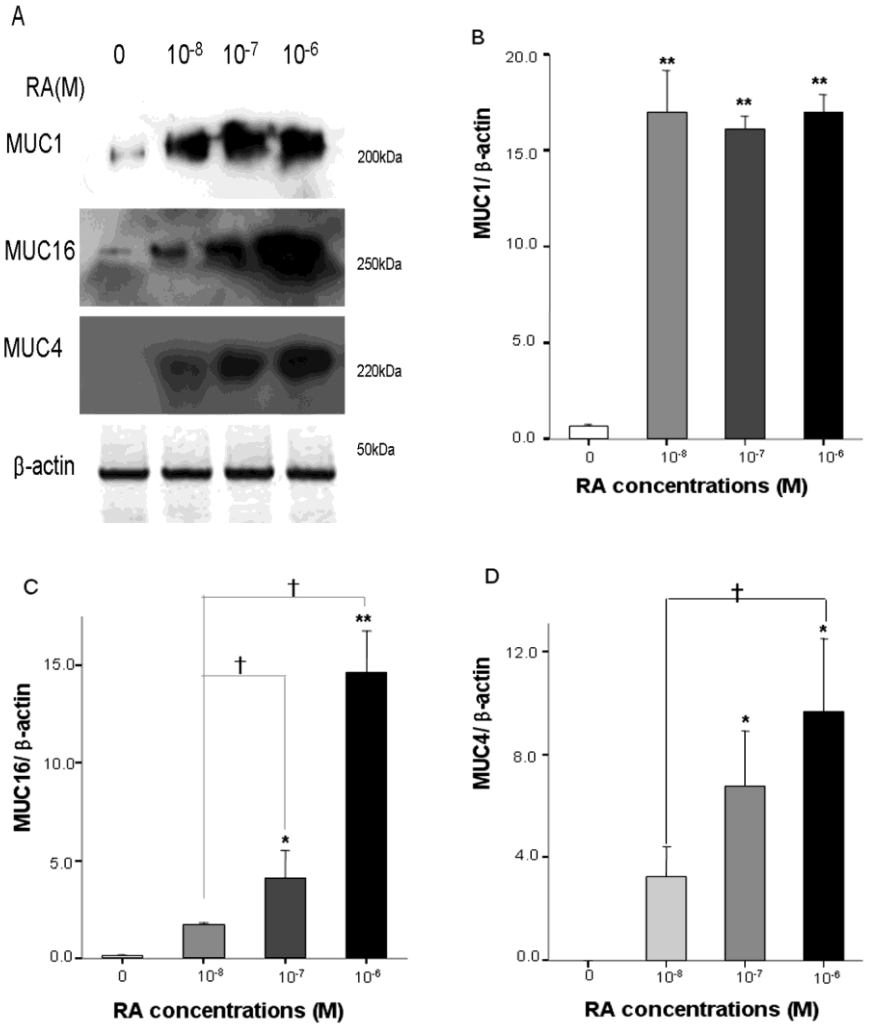

Figure 8. Western blot and densitometric analyses of MUC1, -16 and -4 from corneal epithelial cells cultured in various concentrations of RA. (A) All three mucin proteins were induced after addition of RA. (B) The amount of MUC1 protein did not change with different concentration of RA. (C) MUC16 and (D) MUC4 proteins increased significantly in a concentration-dependent manner in RA-supplemented cultures. (n = 3, *: $p < 0.05$, **: $p < 0.005$ as compared to RA-deficient culture, and †: $p < 0.05$ in comparison between two defined conditions).

IV. DISCUSSION

The aim of this work was to establish fully differentiated culture system for corneal epithelial cell. A serum-free culture method is considered ideal for use in ocular reconstruction surgery and in vitro modeling, and we searched for suitable media for cultivating corneal epithelial cell under defined conditions. Recently, human conjunctival epithelial cell culture method using bronchial epithelial growth media (BEGM) was introduced, and cultured epithelial cell showed features of multi-layered conjunctival epithelium with fully differentiated phenotype.[22] We therefore modified this culture model of human conjunctival epithelial cells to achieve optimally differentiated model for corneal epithelial cell. Retinoid has been known to be essential for epithelial differentiation, and retinoic acid (RA) is used as an essential element of serum-free conditioned media. It has demonstrated modulating effects on cellular proliferation and differentiation in various epithelial tissues including keratinized squamous epithelia in the epidermis as well as mucous epithelia in the airway tract. The existence of epithelial pathogenesis in both hyper- and hypovitaminosis A implies that a critical concentration is required for homeostasis. Previous studies have demonstrated that deficiency of this vitamin can convert secretory epithelium to squamous epithelium, and an excess can convert stratified squamous epithelium to secretory epithelium.[23-28] In this study, we tested the hypothesis that retinoic acid modulates corneal epithelial differentiation. There is also clinical evidence supporting the effect of RA in maintaining healthy ocular surfaces, although little is known regarding how RA works to enhance epithelial health.[32,33] In this regard, we investigated the effect of RA on expression of membrane-associated mucins, which have important roles in maintaining a wet ocular surface. Kruse and Tseng[28] reported that RA stimulated clonal growth of an additional subpopulation in limbal cell cultures, possibly stem cells, and had a dose-dependent effect on corneal epithelial

differentiation in early culture. We further extended their work in that stratified and differentiated epithelial sheets were examined not only for the presence of the differentiation related marker but also for the distribution of positive cells in cultured epithelial cells.

As a result, this study introduced a new culture method for corneal epithelial cells. The cultivated human corneal limbal epithelial cells (HCLE) were 5 to 6 cells thick, well stratified, and differentiated. The phenotypes of cultured epithelial cells were evaluated by immunostaining with antibodies for a proposed stem cell marker, nuclear protein p63, and a corneal epithelial specific differentiation marker, CK3.[34] The results showed strong positive staining for p63 in the basal cell layer, and strong positive staining for CK3 in the suprabasal layer; these staining patterns being similar to those of human corneal limbal epithelium.[34] Interestingly, these markers were differently expressed depending on RA concentration in the culture media. These results suggest that RA has a crucial role in normal cellular differentiation in corneal epithelial cells. We found that deficiency of RA resulted in production of cornified envelope. Because corneal epithelium normally does not express this hyperkeratinized structure in vivo, this finding indicates abnormal terminal differentiation. Interestingly, addition of $> 10^{-8}$ M RA effectively inhibited the formation of cornified envelope, and resulted in a non keratinized stratified epithelial appearance, similar to normal corneal epithelium. This result is in agreement with previously reported findings that abnormal squamous metaplasia is inhibited by RA in various tissues including cultured corneal epithelial cells.[25-28] Higher concentration of RA (10^{-6} M), while effectively inhibiting the squamous differentiation, appeared to induce abrupt basal cell to apical cell differentiation. As a result, cuboidal suprabasal cell and CK3 stained cells were not readily present in epithelial cell sheets cultured in this condition. Based on these morphologic and immunohistochemical results, we demonstrated that corneal epithelial cells from limbal rims could be grown in configurations resembling

those encountered in vivo under serum-free conditions. Concentration of RA seems to have a crucial role in mimicking of physiological differentiation and the higher expression of CK3 in cells grown at 10^{-8} M could be interpreted as an example of this. These cells showed fully differentiated phenotype as demonstrated by strong expression of corneal epithelial cell specific marker, CK3 (Fig.3, Table 1).

Maintaining functional mucous phenotype was another focus of this study. In this regard, 3 membrane-associated mucins (MUC1, -4, and -16), which were known to be expressed in ocular surface epithelium, were immunolocalized in cultured epithelial cells.[15-17] Furthermore, the barrier function of cultured epithelial cells was tested with a rose bengal dye penetrance assay. We chose to use rose bengal to test barrier function, as it has been known to penetrate regions of the ocular surface epithelium in which protection has been compromised, perhaps by alterations in mucins.[29] Recently, Blalock and colleagues demonstrated that protection from rose bengal penetration was provided by MUC16 expression in ocular surface epithelium.[30] Thus, the exclusion of the rose bengal in HCLE cells grown in RA-supplemented media implied that cultured corneal epithelial cells expressed functional MUC16 at their surface. Comparison of expression patterns of the 3 membrane-associated mucins under different concentrations of RA showed interesting features. RA showed a concentration-dependant effect on the expression of MUC16. The epithelial cells cultured in excessive RA (10^{-6} M) expressed MUC16 at all cells except those of the basal layer, whereas those cultured in 10^{-8} M RA expressed MUC16 at apical and subapical cells in a similar pattern to that of normal corneal epithelial tissue (Fig.4). To further characterize mucous phenotype in the cultivated epithelial cells, MUC1 and -4 expressions were also evaluated. Interestingly, MUC1 was mainly expressed at apical cells grown at 10^{-8} M RA, and MUC4 was expressed in the whole cell layers, although staining intensity was relatively weak. These results were comparable to those of native limbal

epithelium (Table 2). Taken together, our results suggest that RA concentrations in the range of 10^{-8} M seem to be the most appropriate for obtaining physiologic phenotypes.

It is not known whether RA regulates membrane-associated mucins in human corneal epithelia. The effects of depletion of vitamin A on ocular surface mucin expression were studied in rats fed on a vitamin A deficient diet.[35] The ocular surface epithelium showed loss of rMuc4 mRNA but not rMuc1 following long term vitamin A deficiency. Similarly, an in vitro culture model using immortalized human conjunctival epithelial cell line showed that addition of RA to culture media upregulated the expression of MUC4 and -16 mRNA, whereas MUC1 was unchanged.[31] In this study, we further investigated the effect of RA in a concentration-dependent manner and in a time-dependent manner. Our data demonstrated that RA upregulated the expression of membrane-associated mucins at both mRNA and protein levels in cultured corneal epithelial cells. Although the data showed the upregulation of all three mucins, the patterns of their regulation were quite different. MUC1 and -16 mRNA were detectable in corneal epithelial cells grown in RA-deficient media, whereas MUC4 mRNA was not. Also, MUC1 protein was detectable in RA-deficient cultures, while MUC 4 and -16 protein was hardly detectable. Despite lack of expression in RA-deficient cultures, MUC4 mRNA significantly induced by RA ($p < 0.05$), and the amount continued to increase in a concentration-dependent manner from 10^{-8} to 10^{-6} M. The expression of MUC1 and -16 mRNA in RA- supplemented cultures also increased significantly compared to those in RA- deficient ones ($p < 0.05$). No significant change was detected in MUC1 mRNA levels among cells grown at different concentrations of RA, whereas the levels of MUC16 mRNA grown at 10^{-8} M RA and those grown at higher concentration of RA were significantly different ($p < 0.05$). These concentration-dependent differences were shown to have a similar pattern to the protein expression. To investigate the time-dependent effects of RA,

cultures were treated with 10^{-8} M RA for different periods. All cultures were grown for 14 days, and RA was added for 0, 3, 7, and 14 days. All 3 mucin mRNAs were upregulated after addition of RA to the culture medium, and the changes showed time- dependency. The amount of induction and the time-dependency were different among the 3 mucins. The greatest induction caused by RA addition was seen with MUC4 mRNA, followed by MUC1, and -16. Our data reinforced previous findings of Hori et al., who also found that addition of RA induced MUC4 and -16 mRNA and protein expression. In contrast to the previous work, however, we found that RA also increased MUC1 expression. There were two possible reasons for this apparent difference. First, we compared the effect of RA in fully differentiated epithelia, whereas Hori and colleagues[31] examined the effect of RA during early differentiation, starting from 0 to 3 days. As a result, epithelial cells grown in RA-deficient media in our study may have been much more differentiated in squamous phenotypes (Fig. 3A). In our study, the difference in MUC1 expression was found to depend on the duration of RA exposure, suggesting that MUC1 expression might be related to the extent of squamous differentiation. Furthermore, no significant differences in MUC1 expression were found among cells grown at different concentrations of RA, and significant induction difference was only seen between RA-depleted and RA-supplemented cultures. Our findings thus partially agreed with the previous report in that MUC1 was consistently expressed in non keratinized epithelium. Second, the aforementioned study used an immortalized conjunctival epithelial cell line, while we used primary corneal epithelial cell. It is not known whether there is a difference in MUC1 regulation between conjunctival and corneal epithelium, but an immunolocalization study showed differing protein expressions in the two neighboring epithelia.[14] Several studies have examined the regulation of mucin expression in various cell lines and tissues, and the data suggest that regulation is epithelium specific. Guzman et al.[36] reported that rMuc1 gene expression from primary rat tracheal epithelial

cells is reduced by RA deficiency, and Shin et al.[37] reported that squamous differentiation, which is induced by RA deficiency, downregulated Muc1 in hamster tracheal epithelial cell. It has been reported that MUC16, another common membrane-associated mucin in ocular surface epithelia, was regulated quite differently after inflammatory cytokine treatment in corneal and conjunctival epithelium.[38] Further study investigating whether membrane-associated mucins are differently regulated in the two ocular surface epithelia would be valuable.

In summary, we report that human corneal epithelial cells were successfully cultured under serum-free, RA-supplemented media. In this study, we demonstrated that RA modulates corneal epithelial differentiation and regulates mucin expression in a concentration- and time-dependant manner.

V. CONCLUSION

This study reported that human corneal epithelial cells were successfully cultured under serum-free, RA-supplemented media. Cultured HCLE cells showed fully differentiated phenotypes with retaining mucin expressions. Our data suggested that such cultured epithelial cells could be an ideal in vitro model for epithelial functional study. Furthermore, they may be suitable for epithelial cell sheet transplantation in ocular surface reconstruction, since they showed similar phenotypes to limbal epithelium. Further studies may be needed to determine the feasibility of this differentiated epithelial cell sheet for ocular surface reconstruction.

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ABSTRACT(IN KOREAN)

인체각막윤부 상피세포 배양조건에서 retinoic acid가 각막상피분화와 점액발현에 미치는 조절효과

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화학화상이나 열화상 또는 스티븐-존슨 증후군과 같은 심한 반흔성 안표면질환은 안표면과 각막윤부에 있는 상피세포의 줄기세포에 심각한 손상이 유발되며, 오랫동안 안과의사들에게 도전의 영역으로 인식되어왔다. 인체외에서 세포배양을 통해 만들어진 각막윤부상피세포편을 이용한 새로운 수술기법은 안표면질환 재건영역에 새로운 전기를 마련하였다. 안표면에는 분비되는 점액과 상층부에서 glycocalyx 구조를 이루는 막성점액이 발현되어 있어 눈물층을 유지하고, 건강한 안표면을 유지하는데 기여한다. 점액층이 상피의 장벽역할에 기여한다는 점을 고려하면, 안표면재건을 위한 이식목적의 각막상피세포편 배양 시 점액발현의 유지가 중요한 요건이 될 수 있다. 그러나, 지금까지 다양한 각막상피세포 배양방법이 소개되었으나, 점액발현이나 기타 장벽기능과 관련된 성상을 연구한 경우는 소수에 불과하다.

본 연구에서는 동물의 혈청을 사용하지 않는 상태에서, 각막상피의 장벽역할이 유지되고 있으며 정상적으로 분화된 각막상피세포편을 얻을 수 있는 배양조건을 확립하고자 하였다. 안표면에서 막성점액은 병원체에 대한 장벽역할을 함으로서 건강한 안표면을 유지하는데

기여하고 있으므로, 배양을 통해 얻어지는 분화된 각막상피세포가 인체내 각막 윤부조직과 유사한 점액발현을 유지하는 것을 목표로 하였다.

비타민 A의 활성물질인 retinoic acid가 상피세포분화와 점액발현에 중요한 역할을 한다는 여러 보고들이 있었다. 이러한 이유로 혈청을 사용하지 않는 배양조건에서 retinoic acid는 세포성장과 분화에 중요한 역할을 담당하는 요소로서 이용되어 왔다. 이러한 사전연구를 바탕으로, 먼저 retinoic acid가 각막상피세포분화에 중요한 조절자로서의 역할을 하는가를 조사하였다. 더불어, retinoic acid 가 각막상피배양조건에서 막성점액발현에 미치는 영향을 확인하고자 하였다.

인체각막상피세포는 각막이식의 공여안의 윤부조직에서 얻었으며, 혈청을 사용하지 않고, 여러 성장요소들이 함유된 배양조건에서 배양되었다. 3세대 배양된 세포들을 Transwell이라는 배양접시에서 배양 후 confluence를 이루면, Air-liquid interface를 만들어 분화를 유도하였다. 각막윤부상피세포를 배양시 retinoic acid를 포함하지 않은 조건과 10^{-9} 에서 10^{-6} M 범위의 다양한 농도의 retinoic acid가 포함된 배양액에서 배양시킨 후, 배양된 각막상피세포를 헤마토실린/에오신 염색을 통하여 세포층의 두께와 모양을 평가하고, 주사전자현미경 검사를 통하여 배양된 각막상피세포의 상층부 구조를 검사하였다. 중층으로 배양된 각막상피편의 분화상태는 각막윤부의 기저부세포를 염색한다고 알려진 p63, 각막상피세포 특이 분화 표지자인 CK3, 그리고, 기능적분화의 지표로서 MUC16의 항체를 이용한 면역조직화학염색 검사를 시행하였다. 또한, 각막상피세포의 장벽역할을 확인하기 위하여 임상에서 흔히 사용되는 rose bengal 염색약을 이용하여 배양된 각막상피세포편을 염색하였다. 마지막으로

retinoic acid가 막성점액의 발현에 미치는 영향을 확인하기 위하여, 농도와 시간을 달리한 조건에서 배양된 각막상피세포를 이용하여 정량적인 real-time polymerase chain reaction과 western blot방법을 실시하였다.

형태학적인 검사에서 retinoic acid 가 포함된 배양조건에서 배양된 각막상피세포편은 중층으로 분화된 비각화 편평상피세포의 모습을 보이고 있었고, 주사현미경 검사상 상피세포의 상부표면에는 수많은 micorvilli구조가 잘 형성되어 있었다. Retinoic acid가 각막상피세포편의 모양에 주는 영향을 농도별로 비교해보면, retinoic acid가 포함되지 않은 조건에서 자란 각막상피세포는 상부에 각화된 층이 두드러지게 나타나는 반면, retinoic acid가 10^{-8}에서 10^{-7} M 정도 포함되면, 각화층도 생기지 않고, 정상적인 비각화편평상피세포의 모습으로 분화되었다. 이러한 배양조건에서는 분화와 관련된 표지자인 p63, CK3, 그리고, MUC16이 인체내 각막윤부조직에서의 발현상태와 매우 유사한 형태로 염색이 되었다. 하지만, 과농도의 retinoic acid (10^{-6} M)가 포함된 배양조건에서는 중층을 이룬 세포층의 수가 적고, 비정상적인 분화형태를 보였다 Rose bengal 염색약을 이용한 장벽기능 검사에서 retinoic acid가 포함된 배양조건에서 배양된 각막상피세포편은 염색약에 투과되지 않았으나, retinoic acid가 포함되지 않은 배양액에서 자란 각막상피세포편은 쉽게 투과되어 염색이 되었다. 이러한 결과는 배양된 각막상피편이 장벽기능을 유지하고 있는 기능적인 MUC16을 발현하고 있음을 시사하는 소견이다. 또한, Real-time PCR과 western blot 방법을 통하여 배양된 각막상피조직에서 세가지 막성점액의 (MUC1, -4, -16) 발현을 확인하였으며, 이들 막성점액이 배양액 내 retinoic acid의 농도와 배양기간 내 노출시간의 영향을 받으면서

발현이 조절되고 있음을 확인하였다.

이상의 결과를 종합하면, retinoic acid가 10^{-8} M 정도 포함된 조건에서 배양하면, 인체내 윤부 조직과 유사한 분화를 보이는 각막상피세포편으로 배양되었다.

결론적으로, 본 연구는 혈청을 사용하지 않고, retinoic acid가 포함된 배양조건을 이용하여 상피장벽의 기능과 막성점액의 발현을 유지하고 있는 생리적인 분화를 이룬 각막상피세포편으로 배양 가능함을 보여주었다. 배양된 각막상피세포는 인체각막상피세포와 유사한 표현형을 가지고 있어, 향후 이식을 위한 각막상피세포편 제작 및 상피세포의 기능연구에 적합한 실험모델이 될 것으로 기대된다. 이러한 각막상피세포 배양조건에서 retinoic acid는 정상 각막상피세포분화에 농도에 의존하는 조절자로서 역할을 하였으며, 세가지 막성점액의 발현에도 농도에 의존하는 영향을 보였다.

핵심되는 말: 각막상피세포, 막성 점액, 비타민 A